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Low concentrations of methamidophos do not alter AChE activity but modulate neurotransmitters uptake in hippocampus and striatum in vitro

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ABSTRACT

Aims: Methamidophos (Meth) is a toxic organophosphorus compound (OP) that inhibits acetylcholinesterase enzyme (AChE) and induces neurotoxicity. As the mechanism of its neurotoxic effects is not well understood, the aim of the present study was to evaluate the effects of Meth on glutamate and gamma aminobutyric acid (GABA) uptake and correlate with cell viability and AChE and Na^+/K^+-ATPase enzyme activities in striatum and hippocampus slices exposed to low concentrations (0.05 to 1.0 μM) of Meth.
Main methods: Hippocampal and striatal slices of rat brain were exposed to Meth for 5 min ([^{3}H]Glutamate uptake) or 15 min ([^{3}H]GABA uptake) for assays. The enzyme activities and cell viability were also accessed at both times in hippocampal and striatal slices and homogenates.
Key findings: At concentrations that did not inhibit AChE, Meth caused changes in glutamate uptake in striatal (0.05 and 1.0 μM Meth) and hippocampal (1.0 μM Meth) slices. GABA uptake was increased by the pesticide in striatum at 0.5 and 1.0 μM and in hippocampus at 0.05 μM. After 3.5 h of Meth exposure, striatal and hippocampal cells showed no changes in viability as well as no inhibition of Na^+/K^+-ATPase were observed after 5 or 15 min exposure to Meth in the same brain structures.
Significance: Results suggest that Meth, even without changing the AChE activity can modify somehow the neurotransmitters uptake. However, further studies are necessary to clarify if this modulation in glutamate or GABA uptake may be responsible to cause some disturbance in behavior or in other neurochemical parameters following low Meth exposure in vivo.

Introduction

Methamidophos (Meth) is a highly toxic systemic organophosphate (OP) used as an insecticide in agriculture against a broad range of insect pests on crops (Deng et al., 2006). Occupational or environmental OP poisoning are characterized by miosis, altered level of consciousness with respiratory failure, vomiting, salivating and breathing difficulty (Lotti, 2000, 2001). On severe cases, convulsion and respiratory arrest followed by death can occur (FAO/WHO, 1993). These symptoms are caused by the inhibition of the primary toxic target of OPs, the acetylcholinesterase (AChE), an enzyme that hydrolyzes acetylcholine (Edwards and Tchounwou, 2005). Acetylcholine is a neurotransmitter (NT) that has functions in both peripheral (autonomic and motor-somatic) and central nervous systems (CNS) and is involved in several brain functions such as muscle activation, memory, neuromodulation and hormonal control (Mesulam et al., 2002; Moretto et al., 2004; Helenius and Lagercrantz, 1974).

OPs phosphorylate the hydroxyl group in a serine residue in the active site of AChE, thus impeding its action on acetylcholine (Costa, 2006). The neurological effects are facilitated due to their great ability to cross the blood brain barrier (Ferrer, 2003). Furthermore, acetylcholine accumulates in the synaptic junction and causes a cholinergic crisis, characterized by continuous stimulation of glands, muscles and the CNS (Drexler et al., 2010). Other neurotoxic effect that has been described is the OP-induced delayed neurotoxicity (OPIDN), characterized by paralysis of the lower limbs, partial sensory loss and degeneration of long axons in the spinal cord and peripheral nerves, evident 10–14 days after exposure. The OPIDN can be attributed to phosphorylation and aging of neuropathy target esterase (NTE) (Glynn, 2000).

Notably, OP can cause some direct effects in some neurotransmitter systems, decreasing gamma aminobutyric acid (GABA) levels and

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increasing dopamine (DA) norepinephrine (NE), serotonin (5-HT) and glutamate (Glu) levels in the brain (Kar and Matin, 1972; Glisson et al., 1974; Ali et al., 1980; Coudray-Lucas et al., 1983; Sivam et al., 1983; Giacobini et al., 1996; Solberg and Belkin, 1997). Moreover, OP can cause a robust activation of glutamate receptors (McDonough and Shih, 1997). Glutamate is the major excitatory neurotransmitter in the mammalian CNS, being important for memory and learning (Izquierdo and Medina, 1997), development and aging (Segovia et al., 2001) and environmental adaptation (Ozawa et al., 1998). However, glutamate may also be a potent neurotoxin whether present at high concentrations in the synaptic cleft, leading to excitotoxicity (Furuta et al., 1997; Danbolt, 2001; Mattson et al., 2002). Controlling this neuronal excitability is one of the roles of the inhibitory neurotransmitter GABA (Mazurkiewicz et al., 1999; Bahena-Trujillo and Arias-Montano, 1999). Some studies have shown that Paraoxon intoxication can alter GABA uptake and its levels in rat brain, triggering seizures when administered in rodents (Kar and Matin, 1972; Mohammadi et al., 2008).

Although many studies tried to clarify neurotransmitter alterations after OP exposure, the results are still inconsistent and, sometimes, contradictory, mainly regarding whether the alterations in glutamate and GABA systems occur before or after the cholinergic alterations. Thus, we hypothesized that low concentrations of Meth that do not affect AChE activity would primarily alter [^{3}H]glutamate and [^{3}H]GABA uptake in slices of hippocampus and striatum of rat brain. Both structures have important functions in brain, hippocampus is a center of learning and memory and striatum is involved in the learning of cognitive and motor activities (Mukai et al., 2010; Fernández-Pérez et al., 2010). In addition, we monitored Na^+/K^+-ATPase activity and cellular viability during the same exposure paradigm in order to verify our hypothesis.

Materials and methods

Reagents

The organophosphate O,S-dimethylphosphoramidotioate (methamidophos, 99%) and the other reagents used were supplied by Sigma-Aldrich Chemical CO (St. Louis, MO). Methamidophos was diluted in water.

Animals

Adult male Wistar rats from our own breeding colony were maintained in an air conditioned room (20–25 °C) under natural lighting conditions with water and food (Guabi-RS, Brasil) ad libitum. All experiments were conducted in accordance with the Guiding Principles for the Use of Animals in Toxicology, adopted by the Society of Toxicology in July 1989. The animals were anesthetized and sacrificed by decapitation. The brain was immediately removed and the hippocampus and striatum separated and placed in the corresponding buffer to each experiment. Hippocampus and striatum slices (0.4 mm) were obtained using a McIlwain tissue chopper to the experiments in vitro.

Acetylcholinesterase activity

Brain AChE activity was estimated by the method of Ellman et al. (1961) with some modifications. We determined AChE activity in homogenates after two different Meth exposure methods. In the first one, we used hippocampus and striatum slices (two slices per well) which were incubated at 37 °C for 5 or 15 min with Tris–HCl 10 mM buffer (pH 7.2, containing 160 mM sucrose and 0.3 μL/mL Triton X-100) and methamidophos at different concentrations (0.05, 0.1, 0.5 and 1.0 μM). Then, slices were homogenized in 100 μL of the buffer described above and centrifuged at 4000 × g for 10 min at 4 °C. The low speed supernatant fraction (S1) obtained by centrifuge was used for AChE activity assays. Further, AChE activity was assayed in S1 fractions exposed to Meth of rat hippocampus or striatum homogenate. The structures were homogenized as detailed above. An aliquot of S1 fraction was exposed to Meth for 5 and 15 min (0.05, 0.1, 0.5 and 1.0 μM). AChE enzymatic assay was performed using acetylthiocholine iodide as a substrate. The rate of hydrolysis of acetylthiocholine iodide was measured at 412 nm for 2 min, through the release of the thiol compound that when reacts with DTNB, produces the color-forming compound TNB. The activity was expressed as % of control.

[^{3}H]Glutamate uptake

Slices (0.4 mm) were obtained by transversal cuts of hippocampus and striatum using a McIlwain chopper. The slices were incubated for 15 min with a HBSS solution containing (mM): 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 5.55 glucose, adjusted to pH 7.2, [^{3}H]glutamate uptake was performed according to Frizzo et al. (1996) with some modifications. Briefly, uptake was carried out at 35 °C by adding 100 μM of unlabeled glutamate and 0.33 μCi/mL [^{3}H]glutamate and methamidophos(0.05, 0.1, 0.5 and 1.0 μM) for 5 min. This time is specific for glutamate uptake and the slices were exposed to OP and substrate simultaneously. The reaction was stopped with 0.5 N NaOH, which was kept overnight. An independent uptake was determined by using choline chloride in the HBSS instead of NaCl, which was subtracted from the total uptake to obtain the Na^+-dependent uptake. Incorporated radioactivity was determined with a Packard scintilator (TRI CARB 2100 TR). All experiments were performed in duplicate.

[^{3}H]GABA uptake

In order to determine the adequate GABA concentration, the assays were based on Schweigert et al. (2005). The slices of hippocampus and striatum were previously incubated for 15 min with a HBSS solution. After, 25 μM GABA and diverse methamidophos concentrations (0.05, 0.1, 0.5 and 1.0 μM) were added and the slices incubated for 15 min. Different from glutamate uptake, 15 min was needed to GABA uptake reaction. Slices were transferred to 24-well dishes and washed with 1.0 mL HBSS. The uptake assay was assessed by adding 25 μM [^{3}H]GABA in 300 μL HBSS and 20 μM of Meth, at 37 °C. Incubation was stopped after 15 min by three ice-cold washes with 1 mL HBSS immediately followed by addition of 0.5 N NaOH, which was kept overnight. Aliquots of lysate were taken for determination of intracellular content of [^{3}H]GABA trough scintillation counting. Na^+ independent uptake was determined by using choline chloride instead of NaCl, being subtracted from the total uptake to obtain the sodium-dependent uptake. The experiments were done in duplicate.

Cells viability

Hippocampal and striatal cell viability were assessed by measuring formazan produced by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) by mitochondrial dehydrogenases (Mosmann, 1983). Slices (0.4 mm) of both brain structures were obtained by transversal cuts of using a McIlwain chopper. Slices were pre-incubated with Meth (0.05, 0.1, 0.5, and 1.0 μM) for 5 and 15 min in oxygenated buffer, containing (in mM): 118 NaCl, 1.2 KH_2PO_4, 4.7 KCl, 2.5 $CaCl_2$, and 1.17 $MgSO_4$. After incubation, the slices were washed twice with 1 mL of buffer. MTT reduction assay was performed in 24-well plates containing 500 μL of buffer, and the reaction was started by adding 0.5 mg/mL MTT. After 30 min of incubation at 37 °C, medium was removed, slices were washed and then dissolved in dimethylsulfoxide (DMSO). The rate of MTT reduction was measured spectrophotometrically at 570 nm.

Na^+/K^+-ATPase activity measurement

The Na^+/K^+-ATPase activity was estimated by the method of Muszbek (1977). The enzyme activity was determined by measuring the amount of inorganic phosphate (Pi) liberated from ATP during the incubation of hippocampal and striatal aliquots. Before, the slices were incubated with Meth (0.05, 0.1, 0.5 and 1 μM) at different times (5 or 15 min). Then, the reaction mixture containing 95 mM NaCl, 15 mM KCl, 1.0 mM ATP (disodium salt), 38 mM Tris–HCl buffer (pH 7.4) was added to aliquot of homogenized slices (50 μg of protein) in a final volume of 0.3 mL. After a 5-min pre-incubation at 37 °C in the presence of 0.1 mM ouabain to specifically inhibit Na^+/K^+-ATPase, the reaction was initiated by addition of ATP and terminated after 15 min of incubation by addition of 1 mL of color reagent (Ammonium Molybdate 2%, Triton X 5% solubilized in H_2SO_4 1.8 M). The released inorganic phosphate was measured spectrophotometrically at $\lambda = 405$ nm. Na^+/K^+-ATPase activity was calculated from the difference between amounts of inorganic phosphate found after incubation in the absence and presence of 1.5 M ouabain.

Protein determination

Aliquots from the homogenized slices and S1 homogenates were separated to protein measurements that were assessed according to Lowry et al. (1951).

Statistical analysis

Statistical significance was assessed by one-way ANOVA, followed by Tukey test for post hoc comparison. Results were considered statistically significant at values of $p<0.05$.

Results

Acetylcholinesterase activity

The AChE activity was not altered in striatum and hippocampus slices and S1 fraction (Fig. 4a and b) by Meth at 5 or 15 min. In S1 fraction of whole homogenized structures, used as an experimental control, the enzymatic activity was also not changed (data not shown). Moreover, we calculated the IC_{50} to Meth in the same brain structures and conditions (slice homogenate and whole structure homogenate at 5 and 15 min, data not shown) and observed that concentrations until 1000× higher than the ones used for assays were responsible to inhibit 50% of AChE activity. The control activity was in hippocampus 0.01726 (5 min) and 0.01774 (15 min) and in striatum 0.1095 (5 min) and 0.0722 (15 min) U/mg of protein.

[^{3}H]Glutamate uptake

Our experiments showed Na^+-dependent [^{3}H]glutamate uptake was increased in both striatal and hippocampal slices system at 1 μM of Meth ($p<0.05$, Fig. 1a and b respectively). However, the same effect could only be observed at 0.05 μM in striatum slices ($p<0.05$, Fig. 1a).

Effect of methamidophos on [^{3}H]GABA uptake

Meth significantly increased [^{3}H]GABA uptake in the striatum (Fig. 2a) and hippocampus slices (Fig. 2b). However, Meth seems to affect differently in each region: in striatum slices, the increase occurred at the higher concentrations used ($p<0.05$, 0.5 and 1.0 μM) while in hippocampal slices at the lower concentrations tested ($p<0.05$, 0.05 μM).

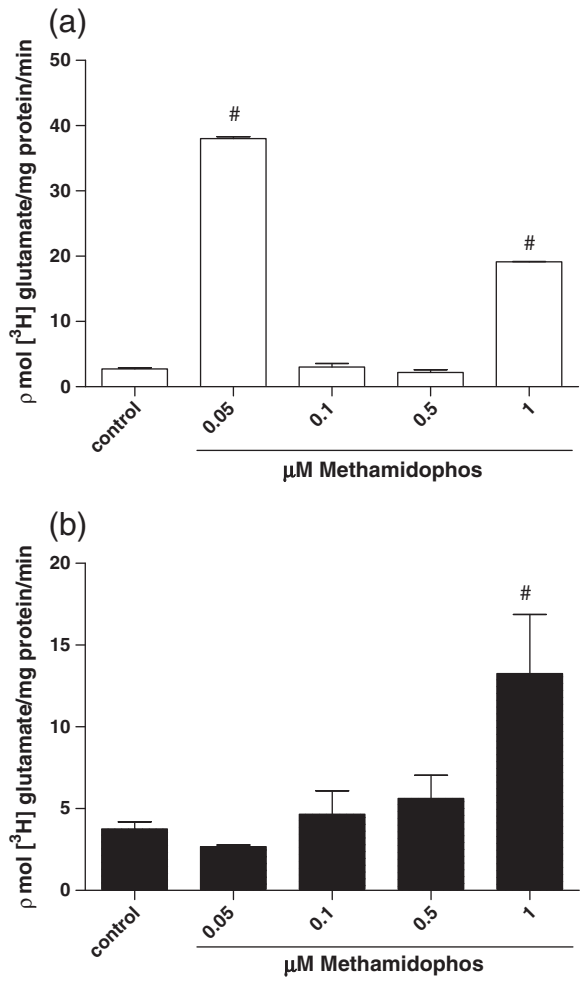

Fig. 1. Effects of methamidophos on [^{3}H]glutamate uptake. Striatum (a) and hippocampus (b). The slices were incubated for 5 min with Meth in different concentrations and [^{3}H]glutamate. Data are presented as pmol [^{3}H]glutamate/mg (protein) and they were analyzed by one-way ANOVA followed by Tukey's multiple range tests (mean ± SEM, n = 8). #Indicates statistical difference from the control, $p<0.05$ to five experiments performed in duplicate.

Mitochondrial viability

We assessed the neuronal viability by MTT assay (Fig. 3). Our results showed that methamidophos did not cause any significant effects on mitochondrial viability of hippocampus and striatum slices at the concentrations tested. A time curve was previously performed to verify the effect of Meth until 3.5 h, the maximal time of control cell viability. None of the exposure times presented significant variation caused by this OP (data not shown).

Na^+/K^+-ATPase activity

The activity of this enzyme is nearly related to neurotransmitter homeostasis and brain synaptic activity (Gloor, 1997). Our results, however, showed no effects of Meth on the Na^+/K^+-ATPase activity (Fig. 5).

Discussion

The use of pesticides has led to serious consequences, in most of the cases conditioned by factors such as high toxicity of the products and improper or lack of collective and individual protective equipment use. This situation is aggravated by the poor socioeconomic conditions and the old-fashioned culture of the majority of

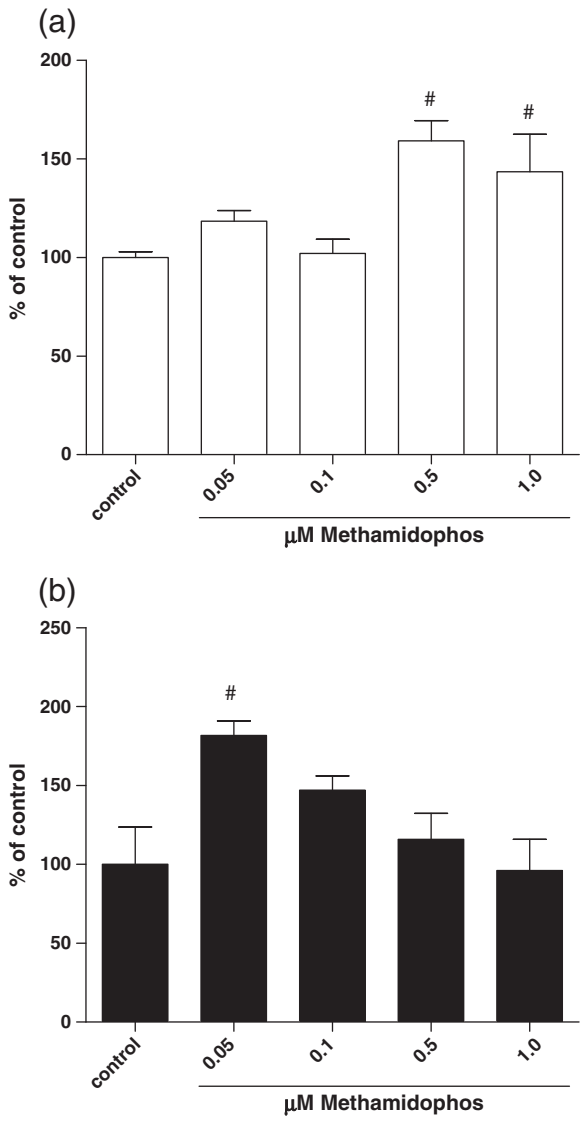

Fig. 2. [^{3}H]GABA uptake in striatum and hippocampus slices after 15 min of Meth incubation. Striatum (a) and hippocampus (b). Data are presented as % of control and they were analyzed by one-way ANOVA followed by Tukey's multiple range tests (mean ± SEM, n = 3). #Indicates statistical difference from the control, $p<0.05$ to three experiments performed in duplicates.

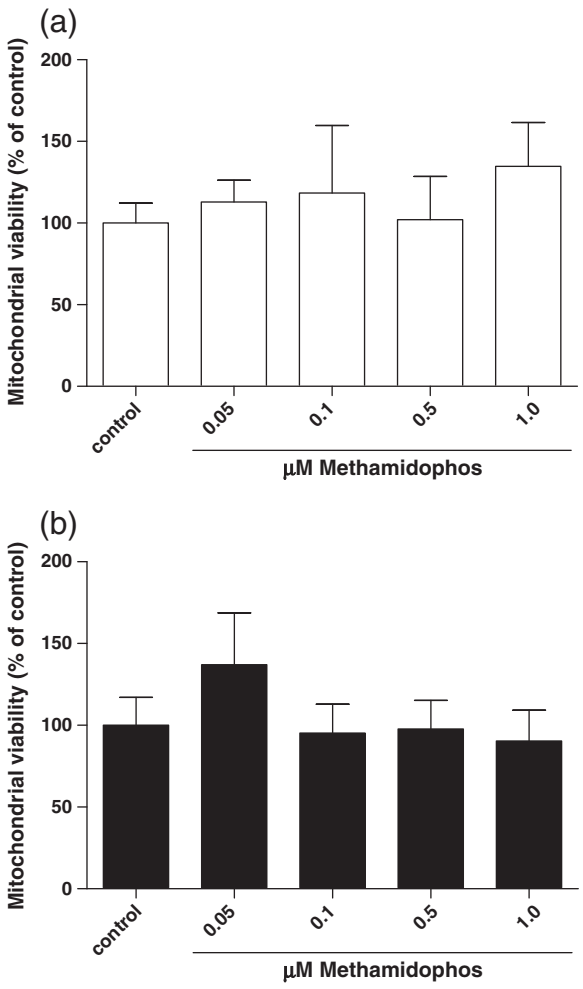

Fig. 3. Mitochondrial viability (% of control) in striatum (a) and hippocampus (b) slices exposed to methamidophos for 3.5 h. Data (mean ± SEM, n = 3) were analyzed by one-way ANOVA followed by Tukey's multiple range tests. No significant differences were observed.

rural workers, increasing their vulnerability to the pesticides toxicity (Silva et al., 2004; Sobreira and Adissi, 2003). Chronic exposure to organophosphate pesticides, even at low doses, can induce neurotoxicity and teratogenic effects (Slotkin et al., 2008). Our in vitro study demonstrated that the neurotoxic effects of methamidophos involve early neuronal alterations that may precede AChE inhibition and cholinergic crisis. This hypothesis is supported by the fact that we observed that low concentrations of Meth alter important neurotransmitter systems without modifying AChE activity and neuronal viability in hippocampus and striatum.

Meth has both hydrophilic and hydrophobic domains and may take advantage of these two opposing forces to easily diffuse through the blood brain barrier resulting in brain AChE inhibition (Singh et al., 1998; Taylor, 2001). In the present study, two different tissue preparations were used: the slice homogenate to represent the same incubation conditions of neurotransmitter uptake and whole structures homogenate just as a control of AChE activity. In the inhibition reaction, the serine hydroxyl group in the enzyme is phosphorylated by the amido(methoxy)phosphinyl moiety of methamidophos with simultaneous loss of the thiomethyl moiety (Thompson and Fukuto, 1982). Several reports in the literature refer AChE as the main toxic target of Meth and that most of the central nervous system alterations are consequences of this enzymatic inhibition (Singh, 2002). On the other hand, using isolated brain structures in order to find more specific mechanisms lead us to find that low concentrations of Meth did not inhibit the brain cholinesterase in vitro but modified [^{3}H]glutamate (Fig. 1a,b) and [^{3}H]GABA (Fig. 2a,b) uptake. These neurotransmitters are particularly important to our study once all glutamate and GABA neuronal innervations cross the hippocampus and striatum, brain areas that play important roles in movement and memory control, functions which are severely affected by OPs (Christie and Blas, 2003; Porras and Mora, 1995).

Alterations in glutamate levels may alter several processes that are dependent on normal levels of this neurotransmitter, such as regulation of the nervous system development (McDonald and Johnston, 1990; Komuro and Rakic, 1993; Johnston, 1995; LaMantia, 1995; Vallano, 1998), neuronal migration (Komuro and Rakic, 1993; Rossi and Slater, 1993), outgrowth of neuronal processes (Pearce et al., 1987; Rajan et al., 1999), normal organization of the somatosensory cortex (Fox et al., 1996) as well as GABAergic activity (Van den Pol et al., 1998). In line with the importance of the glutamatergic signaling, both overstimulation (Johnston, 1995) and understimulation of glutamate receptors are harmful to the developing brain (Ikonomidou et al., 2000). Besides, changes in glutamate uptake can alter glutamate levels, which may influence the levels of

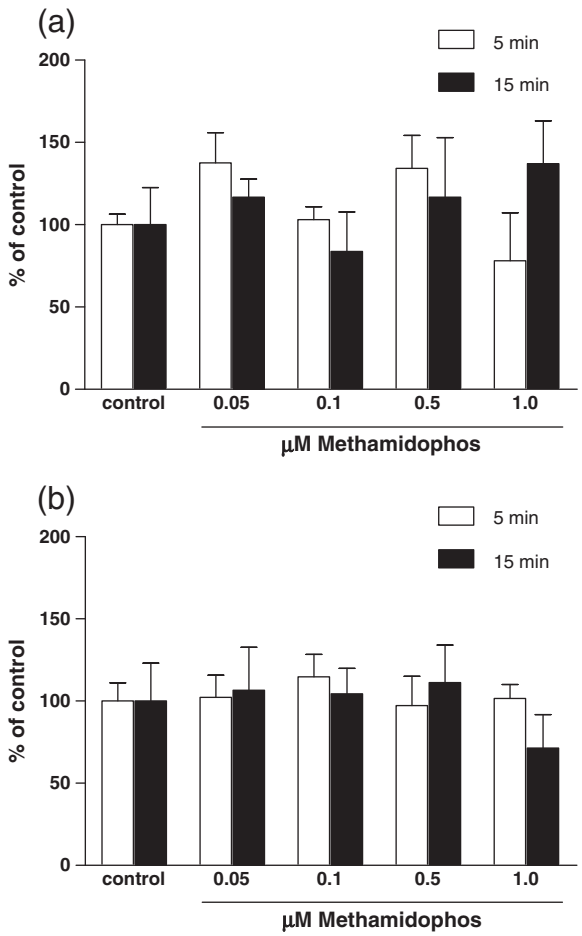

Fig. 4. AChE activity (% of control) striatum (a) and hippocampus (b) slices after Meth exposure. Data (mean ± SEM, n = 3) were analyzed by one-way ANOVA followed by Tukey's multiple range tests. No significant differences were observed.

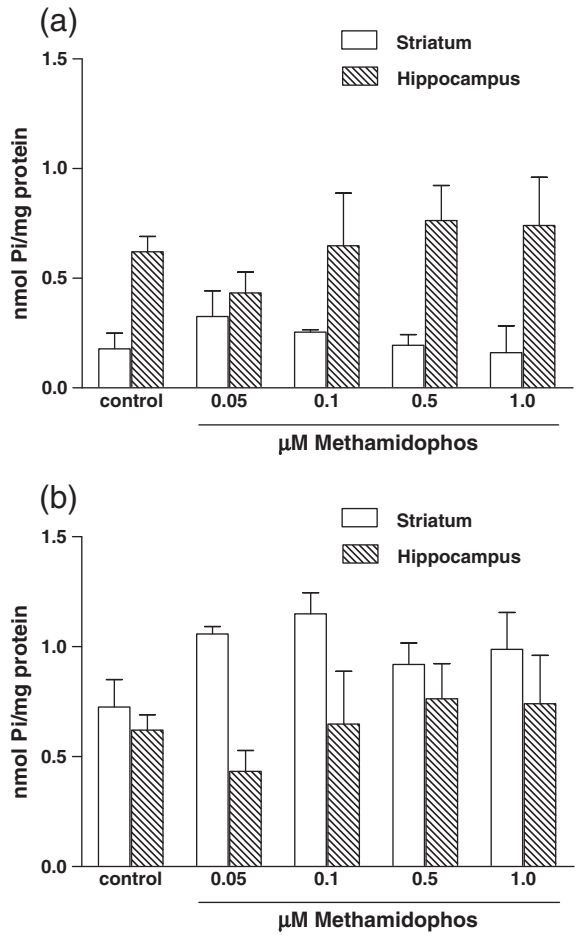

Fig. 5. Na^+/K^+-ATPase activity in striatum and hippocampus slices systems at 5 (a) and 15 (b) min. Values represents means ± SEM, n = 3 which were analyzed by one-way ANOVA followed by Tukey's multiple range tests. No significant differences were found between the control and Meth exposure.

some other neurotransmitters. It is important to emphasize that GABA and glutamate are carefully orchestrated to balance each other, however the major focus of this study is to show the influences of Meth exposure on the different neurotransmitters uptake systems and not to compare the Meth effects in both uptake systems.

Studies of sodium-dependent neurotransmitter uptake in brain slices and homogenates showed that the uptake of glutamate is completely independent on the uptake systems for glutamine, glycine, GABA, taurine, serotonin, catecholamines, dicarboxylates as well as all other neutral and basic amino acids tested (Logan and Snyder, 1972; Balcar and Johnston, 1972). The hypothesis that has been suggested is that glutamatergic nerve terminals may have glutamate transporters and that the degeneration of the axons (either by trans-section of the fiber tracts or ablation of the neuronal cell bodies) leads to an impairment of glutamate uptake activity. Our hypothesis is that Meth, even at low concentrations, can affect some sites by causing oxidative stress, as previously reported in the literature by causing increase in TBARS levels (Kalkan et al., 2009; Ciccheti and Argentin, 2003; Abdollahi et al., 2004). As a consequence, other brain structures would compensate this effect by augmenting glutamate uptake, without causing cellular damage in hippocampus and striatum as hereby demonstrated (Fig. 1a,b).

GABA would be implicated in the convulsive syndrome induced by OP compounds (Shih et al., 1991; Rump and Kowalczyk, 2004). However many studies regarding the relationship between GABA levels or GABA uptake and neurotoxicants are still contradictory. Paraoxon has been reported to decrease GABA uptake in rat cerebellar, cortical and hippocampal synaptosomes (Shahroukhi et al., 2007; Mohammadi et al., 2008). Using Soman in an ex vivo model, Shih and McDonough (1997) have demonstrated an increase in GABA content in various brain regions measured 20 and 80 min post seizure development, indicating increase in GABA release. However, there is no report in the literature regarding GABA homeostasis after Meth exposure. In fact, Camara et al. (1997) reported that Meth did not affect whole-cell currents induced by application of GABA and glutamate to cultured hippocampal neurons, which would suggest that this OP does not affect these neurotransmitters systems. Nevertheless, our experimental model using hippocampus and striatal slices showed an increase on GABA uptake (Fig. 2a,b), which may be caused by a direct effect in the GABA transporters by Meth, triggering the increased glutamate uptake or the opposite, as a consequence of the increased glutamate uptake. As we did not measure the levels of GABA in the studied areas, it is not possible to hypothesize whether this increase in GABA uptake was in response to increased GABA release. It is important to emphasize that our study was in vitro and future studies are necessary to confirm these assumptions. Notably, we could not observe a linear curve concentration–response in the uptake of both neurotransmitters analyzed (Figs. 1 and 2), similar findings were also reported by other authors after OP exposure in vitro (Pourabdolhossein et al., 2009). It may be possible that some concentrations evoke different kind and intensity response reflecting on biphasic results. We believe that this different effect in glutamate uptake between striatal and hippocampal slices could be explained by differences in the density and variety of glutamate transporters in these regions (Beurrier et al., 2009). The difference between glutamate and GABA uptake could be caused by the

differences between the transporters system and their specificities (Borden, 1996). Furthermore, it is difficult to compare our results with other studies in literature, because there is not any study with similar protocol using Meth and neurotransmitter uptake. Some modifications on neurotransmission balance can be caused by a decrease in the cellular viability. Yousefpour et al. (2006) reported that Paraoxon can induce cell death in rat cultured hippocampal cells although our experimental model could not change normal cell viability at low concentrations of Meth in both hippocampal and striatal slices (Fig. 3).

Furthermore, Meth exposure did not interfere with Na^+/K^+-ATPase activity (Fig. 5a,b) in hippocampus and striatum. In the literature, there are few studies relating OP effects on this enzyme, which plays a primary role in regulating membrane function and neurotransmission. Na^+/K^+-ATPase is a membrane-bound enzyme found in most tissues and is primarily responsible for maintaining ionic membrane potential by actively transporting Na^+ and K^+ across cell membranes (Magistretti and Pellerin, 1999). It has been reported that organochloride insecticides can inhibit brain Na^+/K^+-ATPase (Maier and Costa, 1990) but the OP Meth did not present the same potential in our investigation.

Some researchers have identified Meth as the major pesticide used in the Southern Brazil in a population where 75% of the farmers use mainly OP as pesticide (Silva, 1999; Faria et al., 2007). Due to the large risk of these compounds to the population, it is necessary to understand the toxic effects to better inform people about using protective actions against Meth and other OP. Moreover, it is essential that new diagnostic methods of poisoning are used and neurotransmitters uptake may be an alternative especially for detecting previous changes to the characteristic symptoms of severe intoxication.

Although our data is still inconclusive, this in vitro study points out to other possible toxic pathway. The fact that we did not find any alteration in the AChE activity at the low Meth concentrations tested implies that the modifications on neurotransmission system may not be only evocated by AChE inhibition and acetylcholine excess.

Conclusion

Our data showed the uptake alterations of two important neurotransmitter systems followed by methamidophos exposure at low concentrations that did not evoke the major toxicological effect related to OP poisoning, the AChE activity inhibition. We could not observe any changes in cell viability or in the Na^+/K^+-ATPase and AChE activities in slices of striatum and hippocampus. However, we detected that two important neurotransmitters, glutamate and GABA, had their synaptic uptake modified. Our study suggests that AChE inhibition and consequent acetylcholine excess are not the only triggering factor for alterations in GABA and glutamate uptake. Our future directions point out to clarify the mechanisms that cause these uptake alterations here observed by evaluating other neurotransmitters as dopamine and serotonin. In addition, we will investigate glutamate and GABA homeostasis in ex vivo models to further elucidate the toxicological properties of Meth in the central nervous system.

Conflict of interest statement

There are no conflicts of interest.

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